EXHIBIT "A"

11/13/01 -11/27/01
EB on Scaffold Expt: Comparison of RA, TGFB, and Actavin Growth Factors

METHODS:
Preparation of Scaffolds:

1. Sterilized 10 polymer scaffolds overnight in 70% ETOH.
2. Soaked scaffolds in 3 changes of PBS, about 5 mins each change. Transferred with sterilized forceps.

Preparation of EBs:

3. Transfer EBs in media to 50 mL Falcon tube. Pipetted lightly to remove EBs adhered to plate. Washed plate with 5 mL old media.
4. Removed most supernatant. Resuspended in 5 mL new EB media. Transferred to 15 mL Falcon tube.
5. Centrifuged at 800 for 1 minute with brake.
6. Diluted mef trypsin 1:5 (white: 25g porcine tryp/L in 0.9% NaCl) in PBS. Add 2 mL diluted trypsin to the 10mL EB/media mixture. Resuspended by pipetting.
7. Incubated for 5 mins. Added 4mL TNS to cells. Resuspended, and centrifuged at 800 for 3 min. Remove supernatant. Add EB media to aliquot the cells into 2:1 (for scaffolds/EB: EB samples)

Scaffold Conditions:

GF	Stock	Working	# scaffolds	EB Plates w/o Scaff
RA	7.5mg/mL in DMSO	(1:25000)	2	1
TGF-B	2ug/mL	(1:1000)	2	1
Activin	20ug/mL	(1:1000)	2	1
Activin & TGF-B		(1:1000)	2	1
Normal media			2	1

8. Prepared 25mL EB media with GFs. (RA 1:25000; TGF-B 1:1000; Act 1:1000)
9. Added 1mL of respective media to each well to be used for scaffolds. Added scaffolds to wells.
10. Prepared matrigel 1:1 with respective media (50 uL matrigel with each media .
11. Aliquotted cells into eppendorfs. Spin down <1000 for 4 min. Remove supernatant carefully. Add 20-25 uL matrigel-media to cells. Mix well.
12. Removed media from scaffolds in wells.
13. Added 20-25 uL cell-matrigel mixture onto each scaffold.
14. Incubate 30 mins to solidify matrigel.
15. Added 4 mL respective media to each well.
16. For remaining EB's aliquot, spin down and add media. Pipette into wells and add respective media.
17. Placed on shaker.

10 EBs on Scaffold with GF Expt

11/13

Scaffold - PLGA

1. Sterilize 10 polymer scaffolds in 70% ethol overnight
2. Rinse in PBS: Pour out until only 10mL ethol. Pour PBS in small wells. Pour all scaffolds + ethol into plate. Transfer into PBS in 6-well plates with forceps.
3. Transfer 3X for ~5min each

EBs (pyplates)

4. Transfer EBs + media to 50mL Falcon. Pipette lightly to remove EBs stuck to plate. Wash with clean media or old media
5. Remove supernatant until 5mL left. Resuspend in new media (5mL). Transfer to 15mL Falcon. Use same 5mL for all 4 tubes.
6. Repeat again with 5mL. 5. Centrifuge 800 for 1 min with brake.
7. Dilute Trypsin (white, 10X): 1 → 5. Add 2mL diluted to the 10mL EB-media mixture. Resuspend by pipetting. ~~Pour into 2 small petri dishes (5mL each).~~
8. Incubate 3min in the 15mL Falcon

[Dilute Trypsin 1:5 using PBS ⇒ only for EBs]

Scaffold Conditions - 10

1.	RA	2
2.	TGFB	2
3.	Activin	2
4.	TGFB + Activin	2
~~5.~~	~~control~~	~~2~~

EBs w/o scaffold

TGFB
activin
RA
TGFB + activin
⊖ : EB media

(RA: neuronal, Activin + TGF: muscle)

9. Add 25mL BB media in 50mL Falcon. Add GFs:
   RA 1mL / 25mL (should be 1mL / 25mL)
   TGFB 1uL per 1mL media
   Activin 1uL per 1mL media
10. Use 6 well plates (not for tissue culture)
11. Add 4mL TWS to cells. Resuspend. Centrifuge at 800, 3min
12. Prepare matrigel 1:1 with the right media. 5mL of each.

[Cell Counting: use pasteur pipette. Count # cells in 5x5 area. Each square has 16 sq.)

cells × 10^4/mL =

Counted 200 × 10^4 = 2 × 10^6/mL cells × 6mL = 12 million cells

13. Aliquot the cells. Spin down. Add the matrigel-media into the cells. Resuspend.